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Research paper

Isolation of chicken follicular dendritic cells

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Abstract

The aim of the present study was to isolate chicken follicular dendritic cells (FDC). A combination of methods involving panning, iodixanol density gradient centrifugation, and magnetic cell separation technology made it possible to obtain functional FDC from the cecal tonsils from chickens, which had been infected with *Eimeria tenella*. CD45^{-} dendritic cells were selected using the specific monoclonal antibody against chicken CD45, which is a marker for chicken leukocytes, but is not expressed on chicken FDC. Isolated FDC were characterized morphologically, phenotypically and functionally. The phenotype of the selected cells was consistent with FDC in that they expressed IgG, IgM, complement factors C3 and B, ICAM-1, and VCAM-1, but lacked cell surface markers characteristic of macrophages, T-, and B cells. Transmission electron microscopy confirmed their characteristic dendritic morphology. In addition, the identity of the FDC was further confirmed by their ability to trap chicken immune complexes (ICs) on their surface, whereas they did not trap naive antigen (ovalbumin) or ICs generated with mammalian immunoglobulins. Co-culturing allogeneic or autologous isolated FDC with B cells resulted in enhanced B cell proliferation and immunoglobulin production. The lack of MHC restriction, a functional characteristic feature of FDC, further reinforces the identity of the isolated cells as chicken FDC.

Keywords: Follicular dendritic cell; Isolation procedure; CD45; Chicken FDC

1. Introduction

Livestock vaccination remains the most effective method for preventing infectious diseases and reducing the associated economic losses. This is particularly true for avian coccidiosis, a major parasitic disease of substantial economic cost for the poultry industry, caused by parasitic protozoa belonging to the genus *Eimeria*. Infection with *Eimeria tenella* is restricted to the ceca and causes clinical disease. Cecal tonsils are specialized lymphoid structures located in the mucosae at the beginning of each cecum, and provide an important site for contact between the lymphoid system and the cecum contents. Previous reports have demonstrated that during infection caused by *E. tenella*, activation of follicular dendritic cells (FDC) takes place in the cecal tonsils (del Cacho et al., 1993a). Activation of these cells has been shown to be related to acquired immunity to the parasite on the part of the host (del Cacho et al., 1993a).

Abbreviations: FDC, follicular dendritic cells; ICs, immune complexes; GC, germinal centers; MPS, mononuclear phagocyte system; MFI, mean fluorescence intensity.

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As in mammals, avian FDCs are central players in humoral immunity that reside in germinal centers (GC), where they form extensive networks and come into contact with B cells (Szakal et al., 1989; del Cacho et al., 1993a). FDC retain native antigens in the form of immune complexes (ICs) on their surface for presentation to GC B cells (Tew et al., 1982; del Cacho et al., 1995; Gallego et al., 1995). In recent years, considerable progress has been made in understanding the basis of FDC activity by way of studies performed in vitro using purified populations of mammalian FDC (Park and Choi, 2005). The knowledge derived from the in vitro observations has allowed researchers to establish the sequence of GC development in considerable detail. As a consequence, additional critical functions of FDC have been recognized. Moreover, FDC are pivotal in the formation of the GC, by producing anti-apoptotic and growth factors for rapid proliferation of the GC B cells (Qin et al., 1999; Schwartz et al., 1999; Li et al., 2000).

However, despite the important role in driving high-affinity antibody responses, there is a lack of isolation protocols for avian dendritic cells, due to the absence of FDC-specific markers. This situation led us to develop a method to isolate avian FDC. The method described in the present study enabled us to isolate avian FDC, which are $CD45^{-}$ and express IgG on their surface. The FDC identity was confirmed by morphological, phenotypical and functional characteristics. In addition, the isolated FDC maintained their ability to bind ICs on their surface, enhanced proliferation of mitogen stimulated B cells, and augmented antibody production. The protocol described here demonstrates for the first time a novel method for isolating pure avian FDC that will aid in the functional analysis of FDC in avian species and should facilitate future studies on the cellular and molecular interactions within the GC.

2. Materials and methods

2.1. Animals

White Leghorn chickens were hatched and reared coccidia-free under routine laboratory conditions with free access to feed and water. All experiments were performed in accordance with the guidelines approved by the Animal Ethics Committee of our institution.

2.2. Parasite

An *E. tenella* strain was originally obtained from Merck Sharp & Dome (Madrid, Spain). Oocysts were propagated, isolated and sporulated using standard procedures (Raether et al., 1995). Chickens were infected with sporulated oocysts (stored for less than 4 weeks) by oral inoculation into the crop.

2.3. Antibodies

Anti-chicken CD3 (clone CT-3), anti-chicken CD4 (clone CT-4), anti-chicken CD5 (clone 2-191), anti-chicken CD8 (clone CT-8), anti-chicken CD28 (clone AV7), anti-chicken CD44 (clone AV6), anti-chicken CD45 (clone LT40), anti-Bu-1 (clone 21-1A4), anti-chicken IgG (clone G-1), anti-chicken IgM (clone M-4), anti-chicken IgA (clone A-1), anti-chicken MHC I (clone F21-2), anti-chicken MHC II (clone 2G11), and anti-chicken monocyte/macrophage (clone KUL01) were all purchased from Southern Biotech (Birmingham, AL, USA). Anti-chicken CD41/CD61 (clone 11C3) was bought from Serotec (Kidlington, UK). Anti-chicken CD51/61 (clone LM609) was purchased from Chemicom (Hampshire, UK). Anti-human VCAM-1 (clone BBIG-V1) and anti-human ICAM-1 were purchased from R&D Systems (Minneapolis, MN, USA). Anti-chicken complement factor C3 (clone 4D11) and anti-chicken complement factor B were purchased from AntibodyShop A/S (Gentofte, Denmark). Finally, FITC-rabbit anti-mouse IgG, FITC-rabbit anti-goat IgG and isotype controls were purchased from Sigma (St. Louis, MO).

2.4. Lymphocyte isolation

Peripheral blood lymphocytes were obtained from heparinized blood using lymphocyte separation medium (LSM, ICN Biomedicals).

2.5. Follicular dendritic cell isolation

All the chickens received two doses of 5000 *E. tenella* oocysts each, given on days 21 and 28 after hatching. The cecal tonsils were removed on day 8 following the second oocyst administration. The tissue was cut into small pieces (1–3 mm^3) and further incubated in GKN (a solution consisting of PBS supplemented with 11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na_2HPO_4, and 5.5 mM NaH_2PO_4) (Stumbles et al., 1998), supplemented with 0.2% BSA, 1 mg/ml collagenase A (Roche, Mannheim, Germany), and 750 U/ml DNase I (type IV bovine pancreatic; Sigma, St. Louis, MO, USA) at 37 °C for 1 h with constant agitation. The tissue homogenate was then filtered through a 250-µm mesh, resuspended, washed in GKN/5 mM EDTA, and passed through a 70-µm cell strainer (Falcon) to remove clumps of cells. Single cells of cecal tonsils suspended in 10 ml of 1.075 g/ml high density Percoll (Amersham Pharmacia Biotech, Sweden) were centrifuged at 950×*g* for 20 min. The cells were collected from the low density cell layer in order to remove dead cells, red blood cells and cell debris. To isolate cells expressing IgG on their surface, a modified version of the panning technique employed by Wysocki and Sato (1978) was used. A plastic petri dish (92-mm diameter) pre-coated overnight with rabbit anti-chicken IgG (Sigma) (1/50) was washed and incubated with 5×10^7 low density cells at 4 °C for 2 h. Non-adherent cells were discarded and plastic-adherent cells were removed by incubation in 10 ml of EDTA (10 mM in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution) at room temperature for 10 min. Incubation in papain was performed to remove cells retained by the binding of antibodies. Papain (Sigma) digestion was performed in 10 U/ml in PBS (pH 7.4) with 20 mM L-cystein and 20 mM EDTA at 37 °C for 2 h. The reaction was stopped by adding iodoacetamide (Sigma) to a final concentration of 30 mM. Cells collected from dishes with the aid of three washes with PBS were separated into low and high density fractions on iodixanol 60% (w/v) (OptiPrep, Axis-Shield, Oslo, Norway) gradients. Cells were layered by centrifuging over iodixanol discontinuous density gradients at 650×*g* at room temperature for 10 min. Cells in three different iodixanol density gradients (1.069, 1.079 and 1.090 g/ml) were resuspended in cell suspension medium (0.85% NaCl, 10 mM Tricine–NaOH, pH 7.4) according to the manufacturer's guidelines. The low density cells were collected, resuspended and washed in RPMI 1640/5 mM EDTA. Cells were incubated with mouse anti-chicken CD45 (Southern Biotech) at 1/100 dilution for 45 min, washed, and incubated with rat anti-mouse IgG coated Dynabeads (Invitrogen, Paisley, UK). Bead-bound cells were discarded according to the manufacturer's guidelines, and the remaining unbound cells were transferred to a fresh tube and washed in RPMI 1640/5 mM EDTA. Final cell viability as determined by the trypan blue dye exclusion test, was >90% for all tissue samples. Isolated cells were cultured in RPMI 1640 (Sigma) supplemented with 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 40 μg/ml gentamicin, 100 IU/ml penicillin; 100 μg/ml streptomycin and 10% chicken serum. Chicken serum was heat-inactivated in a 56 °C water bath for 30 min to inactivate complement components before use.

2.6. Light and transmission electron microscopy

For the light microscopy studies, 1×10^4 isolated FDC were placed into a cytobucket, centrifuged onto poly-L-lysine coated glass slides (Sigma), and then visualized using a phase-contrast microscope (Nikon Elipse). For the electron microscopy studied, isolated cells were fixed in 1% glutaraldehyde at room temperature for 60 min, and further fixed in 1% osmium tetroxide for 30 min. After final washing in buffer and dehydration in ethanol, fixed cells were cleared in propylene oxide and embedded in Epon-Araldite (1/1) (BioRad, Hemel Hempstead, UK). Ultrathin sections (40–60 nm) were prepared and stained with uranyl acetate and lead citrate.

2.7. Flow cytometry

Isolated FDC (5×10^4) were incubated in normal serum (Vector Lab.) for 10 min, followed by incubation in 0.2 ml of the primary antibody or isotype control at 1/100 dilution for 90 min. After three washes, a second incubation was carried out in the appropriate fluorochrome conjugated secondary antibody at 1/100 dilution for 30 min. Staining was analyzed using an EPICS Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with an argon ion laser set at 488 nm with 20 mW of power to excite fluorescein. Fluorescein-isothiocyanate (FITC) fluorescence was determined at 525 ± 15 nm with a band-pass filter. A total of 20,000 gated cells, on the basis of forward-and side-light scatter, were analyzed for each sample. Each experimental run included positive and negative controls which were used to calibrate the region necessary to discriminate the labeled cell populations from the background debris. Data files were analyzed with software provided with the EPICS Elite flow cytometer (version 4.02, Coulter Electronics).

2.8. Immunofluorescence technique

Isolated cells were brought to a concentration of 1×10^4 cells/ml. The cell suspension was centrifugated for 2 min at 800 rpm (Cytospin 3, Shandon) onto slides coated with 0.1% poly-L-lysine (Sigma). Immediately after centrifugation, the cells were incubated in cold acetone (−20 °C) for 20 min followed by three washes in PBS (5 min each). The cells were then incubated in normal horse serum (blocking reagent) (Vector Lab., Burlingame, CA, USA) for 10 min, followed by incubation for 90 min with one of the primary antibodies (anti-VCAM-1, anti-ICAM-1, anti-chicken complement factor C3, and anti-chicken complement factor B) or isotype control. After washing in PBS, the slides were incubated for 30 min with the appropriate secondary antibody conjugated to FITC. After washing in PBS, the slides were air-dried, embedded in a mounting medium (Crystal Mount aqueous, Sigma), and covered with a coverslip.

2.9. Ability to bind immune complexes

2.9.1. FITC-ovalbumin uptake

In order to measure the efficacy of antigen and IC uptake by the isolated FDC, we added fluorescein conjugated ovalbumin (FITC-OVA, Invitrogen) as antigen or IC to cultures of isolated cells. To generate ICs, FITC-OVA was incubated with mouse IgG anti-OVA or chicken IgG anti-OVA at a ratio of 1 μg of OVA to 25 μg of IgG in PBS, at 37 °C for 30 min (de Jong et al., 2006). ICs were collected by precipitation and FITC-OVA concentration was measured with a scanning multi-well spectrophotometer at a wavelength of 450 nm. Isolated FDC were incubated with FITC-OVA as antigen or IC at both 39 °C and 4 °C for 50 h. After the addition of 100 μg/ml FITC-OVA to 10^6 isolated cells in 3 ml of culture medium, we took samples at different time points to measure the FITC-OVA content in the extracellular fluid. Samples were centrifuged gently to remove cells and stored at −20 °C prior to analysis. After all the supernatants had been collected, we transferred them to a 96-well ELISA plate and the FITC-OVA content was quantified by fluorimetry. Fluorescence was measured with an excitation wavelength of 490 nm and an emission wavelength of 535 nm. To calculate the uptake of FITC-OVA by single cells, FACS analysis was performed at comparable time points and the mean fluorescence intensity (MFI) was determined semi-quantitatively.

2.9.2. Immunoelectron microscopy

Isolated FDC were incubated with either FITC-OVA, chicken ICs, or mouse ICs (the ICs were generated as described in Section 2.8) at 39 °C for 50 h. After these three separate incubations, the cells were fixed, embedded and ultrathin sections prepared as described in Section 2.6. As a marker of the cells that had bound FITC-labeled antigen (ovalbumin) on their surface, a monoclonal anti-FITC (Clone FL-D6, Sigma), which reacts with conjugated FITC, was used. Sections were incubated with the monoclonal anti-FITC at 4 °C overnight. The sections were then incubated with the secondary antibody (biotinylated anti-mouse IgG, Vector Lab.) at room temperature for 90 min, followed by incubation with avidin-biotinylated peroxidase complex (Vectastain Elite kit, Vector Lab.) at room temperature for 90 min. The binding sites of the primary antibody were visualized with diaminobenzidine tetrahydrochloride and hydrogen peroxide. The sections were counterstained with uranyl acetate and lead citrate.

2.10. B cell proliferation

Isolated FDC were tested for their ability to induce B cell proliferation in a colorimetric BrdU immunoassay (Cell Proliferation BrdU ELISA, Boehringer-Mannheim). Briefly, the assay was based on colorimetric detection (absorbance reading in a scanning multi-well spectrophotometer) of anti-BrdU antibody, coupled with peroxidase, reacting with a tetramethylbenzidine substrate. Peripheral lymphocytes (5×10^5) were cultured with 10 ng/ml LPS in the presence or absence of an increasing number of autologous or allogeneic isolated FDC. The lymphocyte:isolated FDC ratios used were 1000:1, 200:1, 100:1, 50:1 and 25:1. The proliferation assay was carried out in round-bottomed wells of microtiter trays containing 0.2 ml of RPMI. BrdU was added to the culture on day 4, and proliferation measured on day 5 using the ELISA methodology according to the manufacturer's instructions. Control wells contained medium with either lymphocytes or isolated FDC.

2.11. Antibody assay

ELISPOT assay was carried out in order to quantify IgG-secreting cells. Lymphocytes (5×10^5) were cultured with 10 ng/ml LPS, in the presence or absence of autologous or allogeneic (5×10^3) isolated FDC, in 0.2 ml of RPMI for 10 days. Microplates were coated with the capture antibody, anti-chicken IgG (Sigma), at 4 °C overnight, blocked with bovine serum albumin (BSA) (10 g/l in PBS containing 0.05% Tween 20 (PBS/T)) at room temperature for 2 h, and washed three times with PBS. ELISPOT assays were then performed adding isolated FDC and lymphocytes. Following the co-culture of the cells for 24 h, the plates were washed three times with PBS and three times with PBS/T. Biotinylated anti-chicken IgG detection antibody was added and incubated for 24 h at room temperature. The plates were washed four times with PBS/T and subsequently incubated with the third reagent, horseradish peroxidase-conjugated streptavidin (Dako, Denmark), at a 1/2000 dilution in PBS/T containing 1% BSA at room temperature for 2 h. Plates were washed three times with PBS/T and three times with PBS and the spots were developed using 200 μl of a solution consisting of 3-amino-9-ethylcarbazole (Pierce Pharmaceuticals, Rockford, IL; 10 mg/ml in *N*,*N*^{9}-dimethylformamide), freshly diluted 1/30 in 0.1 M sodium acetate (pH 5.0). The solution was filtered, and H_2O_2 added to a final concentration of 0.015%. The plates were developed at room temperature for 10–30 min until the spots became visible macroscopically, after which the reaction was stopped by rinsing with distilled water. Colored spots, indicating the IgG-secreting cells, were observed using a stereomicroscope and accurately counted using the trace facility of an image analyzer (IM50 Leica).

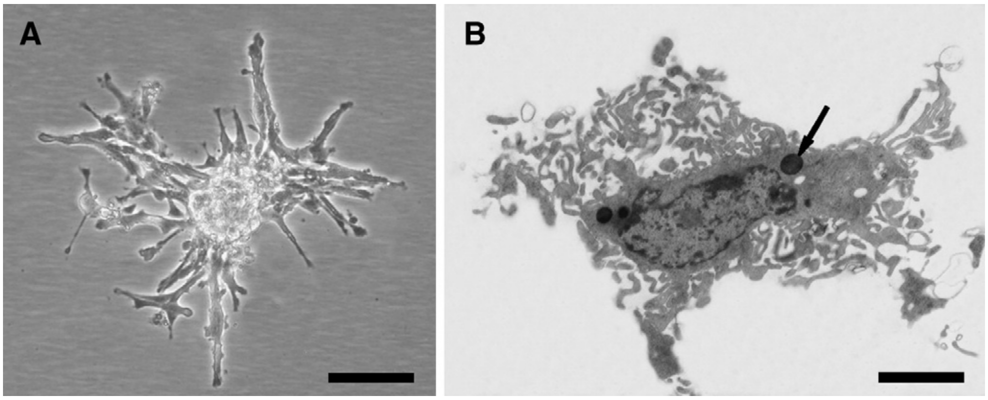

Fig. 1. Morphological characteristics of isolated FDC observed under a phase-contrast microscope (A) and a transmission electron microscope (B). (A) A typical isolated FDC, stellate in shape, sending out long, thin cytoplasmic processes. Bar = 8 µm. (B) Transmission electron micrograph illustrating an isolated FDC showing numerous long, thin convoluted dendrites on both the cellular body and the cytoplasmic processes. Note the electron-dense cytoplasmic granules (arrow). Bar = 4 µm.

Control wells contained medium with either lymphocytes or isolated FDC.

3. Results

3.1. Morphology and phenotype of isolated FDC

Using the isolation method reported here, we obtained 5.2×10^5 FDC per chicken at about 92.3% purity. Isolated FDC showed an average viability of 89.7%, based on the ability of viable cells to exclude trypan blue.

When viewed by phase-contrast microscope, isolated FDC showed a stellate morphology, with large, thin, and slightly branched cytoplasmic processes, extending in many directions from the cell body (Fig. 1A). When looked at with a transmission electron microscope, isolated FDC exhibited numerous filiform and convoluted dendritic processes, on both the cellular body and the cytoplasmic processes. The cytoplasm contained electron-dense granules around the nucleus (Fig. 1B). In the cultures, isolated FDC were aligned in rows with the cytoplasmic processes of the cells extended to contact the processes from both neighboring cells (Fig. 2).

The population of isolated FDC was further characterized to determine whether the cells had phenotypic characteristics consistent with those of avian FDC. Using the monoclonal antibodies listed in Table 1, we

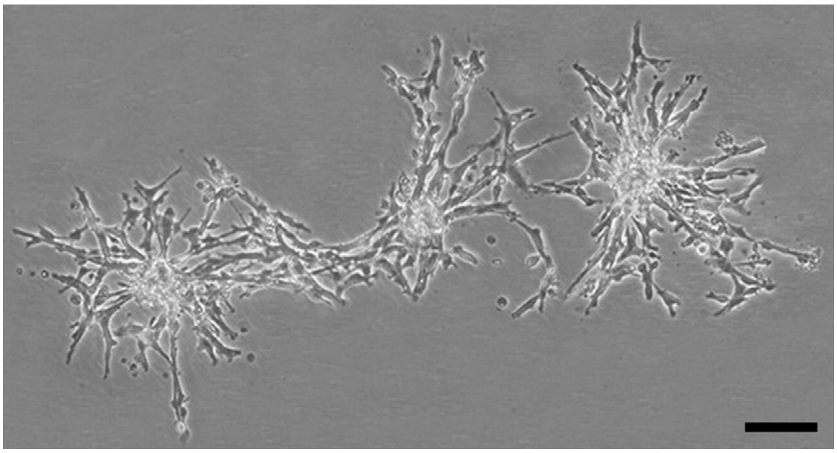

Fig. 2. Alignment of the isolated FDC in rows after culturing for 10 days, observed under a phase-contrast microscope. The processes extending from one cell connect with the processes from both neighboring cells. Bar = 10 µm.

Table 1
Monoclonal antibody reactivity with surface markers of isolated cells

Determinant	mAb	Fluorescence intensity
Leukocyte markers		
CD44	AV6	−
CD45	LT40	−
MHC molecules		
MHC II	2G11	−
MHC I	F21-2	−
T cell markers		
CD3	CT-3	−
CD4	CT-4	−
CD5	2-191	−
CD8	CT-8	−
B cell markers		
CD28	AV7	−
Bu-1	21-1A4	−
Immunoglobulins		
IgM	M-4	++
IgG	G-1	++
IgA	A-1	−
Complement factors		
Factor C3	4D11	++
Factor B	135-LTD	++
Macrophages		
Monocytes/macrophages	KUL01	−
Adhesion molecules		
CD41/CD61	11C3	+
CD51/CD61	LM609	+
VCAM-1	BBIG-V1	++
ICAM-1	BBA17	++

Each plus represents about one log increase over background.

found that the isolated FDC were negative for leukocyte, macrophage, T- and B cell markers, whereas they expressed FDC associated molecules including IgG, IgM, complement factors C3 and B, and the adhesion molecules VCAM-1 and ICAM-1 (Figs. 3 and 4).

3.2. Ability to bind immune complexes

The uptake of FITC-OVA, as antigen or IC, was measured using a fluorimetric analysis to determine the extracellular protein concentration in the culture medium (Fig. 5A). In parallel, FACS to measure the FITC intensity in isolated FDC cultured with FITC-OVA was carried out (Fig. 5B). Our results showed that the uptake of FITC-OVA took place exclusively when the protein was administered in the form of ICs generated with chicken IgG anti-OVA (Fig. 5). We found a progressive decrease in the FITC-OVA (as chicken ICs) concentration in the culture medium as the fluorescent intensity in the cells increases (Fig. 5) indicating that the ICs that were generated with chicken IgG anti-OVA were trapped by the cells from the culture medium. Although ICs were taken up by the cells during the first 30 h of culture the great majority of this uptake had taken place by 20 h. Upon addition of chicken ICs to the culture medium, an uptake of 234 µg of FITC-OVA/10^6 isolated cells was achieved after 30 h at 39 °C. The amount of protein (ovalbumin) taken up per isolated FDC was calculated to be 0.23 ng. Note that neither FITC-OVA alone nor as IC generated with mouse IgG anti-OVA, were taken up by the isolated FDC (Fig. 5). Uptake of antigen (FITC-OVA) or ICs did not take place at 4 °C (Fig. 5), indicating an active process rather than passive attachment of the protein to the cells.

Immunoelectron microscopic examination revealed a positive reaction on the surface of the cells incubated with the antigen plus chicken IgG anti-OVA. Positivity was found as a strong immunoreaction product on the plasma membrane of the cell body, processes, and filiform dendrites (Fig. 6). The ICs gave a dot reaction that outlined the cell (Fig. 6). No

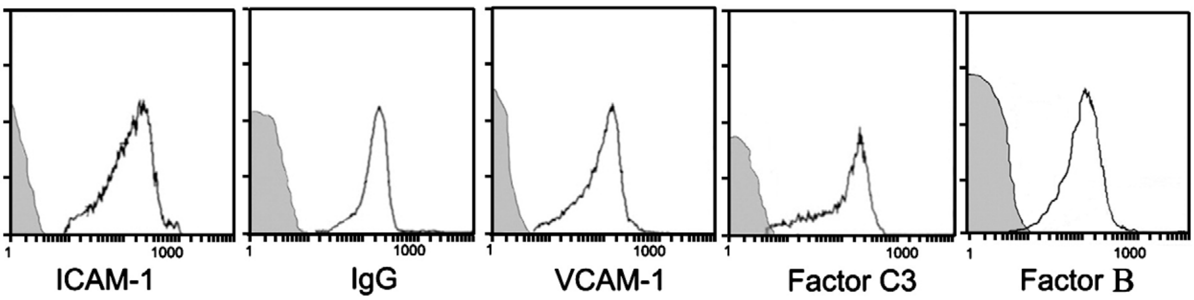

Fig. 3. Flow cytometry analysis of isolated FDC incubated with monoclonal antibodies against FDC associated molecules. The histograms show the fluorescent intensity of the ICAM-1, IgG, VCAM-1, factor C3, and factor B expression on the isolated FDC. The filled histograms show the background fluorescent levels of appropriate isotype controls. The results are representative of three experiments with similar results.

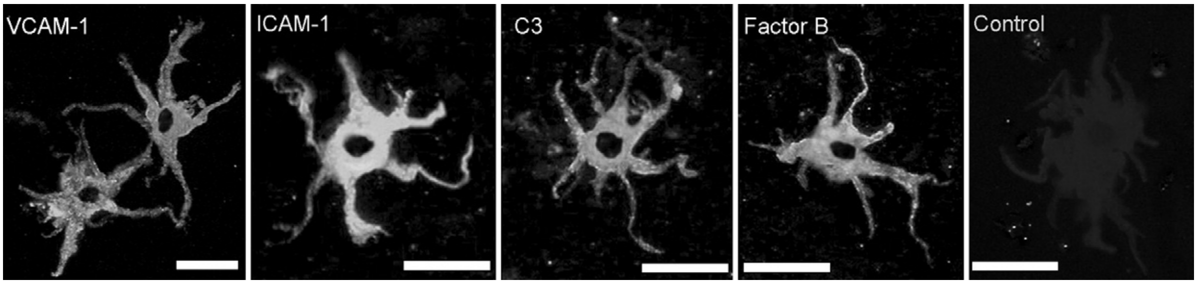

Fig. 4. Detection of cell surface markers using monoclonal antibodies. Expression of VCAM-1, ICAM-1, factor C3, and factor B on the surface of the isolated cells, observed by immunofluorescence microscopy. Bar = 10 μm.

detectable immunostaining was observed on the cells incubated with either ovalbumin, or ICs generated with mouse IgG anti-OVA.

3.3. B cell proliferation

The proliferation of autologous and allogeneic B cells was quantified at different lymphocyte:isolated FDC ratios by the measurement of BrdU incorporation. As increasing numbers of isolated FDC were cultured in the presence of a fixed number of lymphocytes, BrdU incorporation increased linearly. The assay showed a high correlation ($r^2 = 0.97$) between the number of isolated FDC placed into each well and the amount of BrdU that was incorporated (Fig. 7). Differences in the amount of BrdU incorporated by lymphocytes cultured with autologous or allogeneic isolated FDC were not observed. Controls, which consisted of either lymphocytes or isolated FDC incubated with LPS, did not show BrdU incorporation (Fig. 7).

3.4. Induction of IgG synthesis

A representative experiment of an ELISPOT assay to determine the number of IgG synthesizing cells is show in Fig. 8A. The IgG spots were clearly visible when

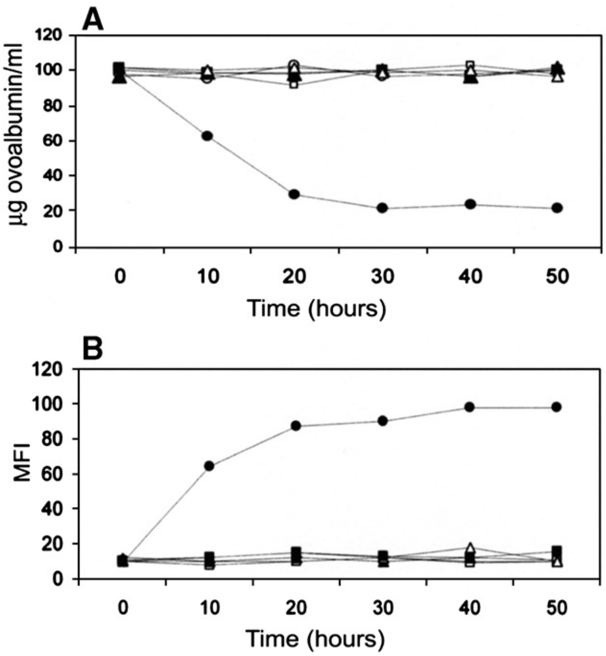

Fig. 5. Uptake of FITC-OVA, as antigen (▲), or ICs by the isolated FDC at 39 °C (black symbols) or 4 °C (white symbols). The ICs were generated by incubating FITC-OVA with either mouse IgG anti-OVA (■) or chicken IgG anti-OVA (●). In order to prove the protein uptake, the protein content in the culture medium was measured by fluorimetry at different time points (A) and the cells were analyzed for FITC expression by FACS (B). The data shown are representative of three experiments.

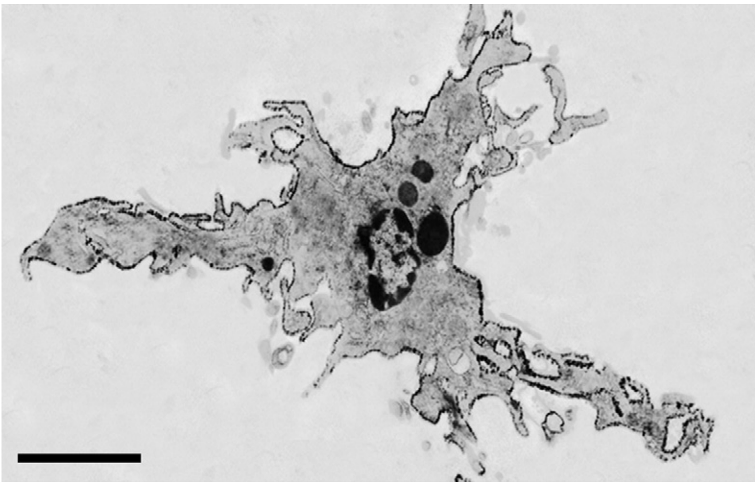

Fig. 6. Immunoelectron micrograph of an isolated FDC, showing a positive reaction for the antigen (OVA) in the form of IC, which were generated with chicken IgG anti-OVA. Positivity is observed as a dotted pattern of staining on the surface of the cell body, processes and convoluted dendrites. Bar = 7 μm.

lymphocytes were cultured with isolated FDC plus LPS, whereas no spots were observed when lymphocytes were cultured in the absence of isolated FDC or LPS (Fig. 8A). The statistical analysis showed no significant differences in the production of IgG synthesizing cells when lymphocytes stimulated with LPS were cultured in the presence of autologous or allogeneic isolated FDC. The spots had a defined circular morphology that could easily be counted by the ELISPOT image analyzer (Fig. 8B,C).

4. Discussion

This paper describes, for the first time, a novel method for the isolation of chicken FDC from cecal tonsils. Cell fractionation was performed using a combination of panning, iodixanol density gradient centrifugation, and magnetic cell separation technology. As in mammals, germinal centre FDC in chickens are known to take up and retain ICs on their surface (Vervelde et al., 1993; del Cacho et al., 1995; Gallego et al., 1995). Using the panning method, we isolated cells expressing IgG on their surface, namely FDC and B cells, and removed non-expressing IgG cells. Microscopic examination showed that positively selected cells were dendritic (FDC) or round (B cells) in shape. Metrizamide density gradient centrifugation has been successfully used to isolate cells, which have dendritic morphology and are capable of antigen presentation, from human, murine, equine, pig and bovine tissues (Renjifo et al., 1997; Siedek et al., 1997; Makala et al., 1998; Peña-Cruz et al., 2001). Applying density gradient centrifugation, we obtained a purer isolate of the cells with dendritic processes and expressing IgG on their surface. To avoid contamination by other cells, we used magnetic bead separation to

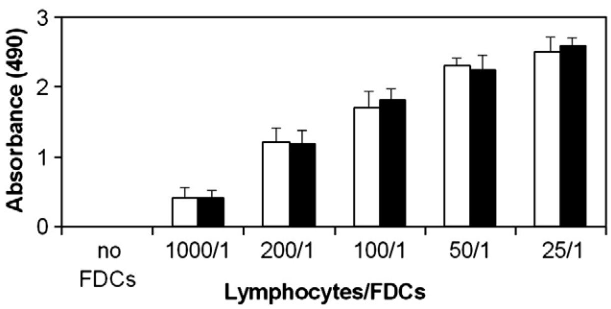

Fig. 7. Proliferation of B cells stimulated with LPS and cultured with increasing numbers of isolated FDC with ratios from 1000:1 to 25:1. Proliferation was measured by BrdU incorporation which was quantified as absorbance. B cells were cultured with either allogeneic (white bars) or autologous FDC (black bars). One of three experiments with similar results is shown in this figure.

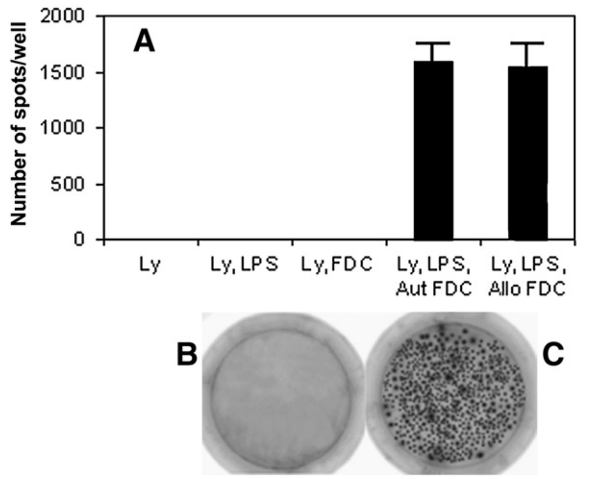

Fig. 8. Detection of IgG-secreting cells by ELISPOT. Lymphocytes, stimulated or non-stimulated with LPS, were cultured in the presence or absence of autologous or allogeneic isolated FDC (A). The results are representative of three experiments with similar results. Representative ELISPOT wells are shown after plate development. (B) Lymphocytes non-stimulated with LPS and incubated with autologous isolated FDC. (C) Lymphocytes stimulated with LPS and incubated with autologous isolated FDC.

isolate $CD45^-$ cells. CD45 is a transmembrane glycoprotein expressed on all chicken leukocytes (granulocytes, monocytes–macrophages, T-, and B cells) (Jeurissen et al., 1988), but it is not expressed on FDC (Muñoz-Fernandez et al., 2006). Using the combination of techniques described above, we isolated $CD45^-$ cells that both expressed IgG on their surface and had dendritic morphology in the 99% purity range, as confirmed by flow cytometry and transmission electron microscopy. The vast majority of $CD45^-$ isolated cells showed morphological and ultrastructural features consistent with those reported for chicken FDC (Olah and Glick, 1979; del Cacho et al., 1992, 1993b). $CD45^-$ isolated cells were dendritic in shape, exhibited convoluted dendrites extending from both the cell body and cytoplasmic processes, and contained several electron-dense cytoplasmic granules. However, despite the fact that the dendritic morphology and electron-dense cytoplasmic granules were typical for chicken FDC, we assessed FDC identity by phenotypic characterization using a panel of monoclonal antibodies. The phenotype of the isolated cells with dendritic morphology was consistent with FDC, in that they expressed IgG, IgM, complement factors C3 and B, ICAM-1, and VCAM-1. Our results are in agreement with the studies by van Nierop and de Groot (2002) and Chaplin and Zindl (2006), demonstrating that ICAM-1 and VCAM-1 are expressed in significant amounts on FDC.

Although FDC bind ICs on their surface, it is known that they do not internalize, process, and present antigens in the context of MHC II molecules (Sukumar et al., 2006), but, rather, present native antigen in the form of ICs to B cells. In line with this, using immunoelectron microscopic techniques, we demonstrated that the isolated cells bound ICs on the surface of their cell body, cell processes, and convoluted dendrites. In addition, ICs were not seen within the cytoplasm, which is indicative that the cells did not endocytose ICs. These results, together with the finding that the isolated cells were negative for the leukocyte markers that recognize surface molecules on macrophages, B- and T cells, reinforces the view that the cells isolated in the present study are FDC. Interestingly, using fluorimetry techniques, we found that isolated cells did not uptake either antigen (ovalbumin) or ICs generated with mammalian Ig (mouse IgG anti-OVA). These results further support the suggestion that the cells isolated in the present study are FDC. Cells belonging to the mononuclear phagocyte system (MPS) internalize antigen, whether native or in the form of ICs, through several surface receptors (Stossel, 1999; Qureshi et al., 2000; Bliss et al., 2005). However, by contrast, FDC do not internalize native antigen, but instead trap ICs on their surface by Fc and complement receptors. It is well known that chicken Fc receptors specifically bind chicken immunoglobulin, but they do not bind mammalian Ig (Duncan and McArthur, 1978; Wick et al., 1982). In the present study, in which the complement was absent from the culture medium, the isolated cells bound chicken ICs, but they did not uptake native antigen or mouse ICs. As a consequence, it is conceivable that all these isolated cells are FDC, and are not constituents of the MPS.

In order to test the functional activities of the isolated cells, B cell proliferation and ELISPOT assays to quantify Ig-secreting cells were performed as markers of the stimulatory capacity of the isolated cells. We found that both allogeneic and autologous isolated cells enhanced proliferation of B cells and also led to increased production of immunoglobulins. Therefore, B cells were stimulated in an MHC unrestricted manner. Consequently, isolated cells were capable of stimulating proliferation and antibody production in allogeneic B cells. Furthermore, the lack of MHC restriction, a functional characteristic feature of FDC (Fakher et al., 2001), is in marked contrast to the activity of the common accessory cells that present antigen to T cells.

The present isolation method overcomes the difficulty of obtaining pure chicken FDC in a functional state, and facilitates further in vitro studies to extend our understanding of the microenvironment in which FDC develop their function. The present isolation method does indeed allow FDCs to be obtained from normal uninfected chickens. However, in these circumstances, the number of cells obtained ($(7–10)\times10^3$ FDC per chicken) was not enough to carry out the characterization tests and, since this is the first time that chicken FDCs have been isolated, the characterizations tests were essential to demonstrate that the isolated cells were unquestionably FDCs. As *E. tenella* activates the lymphoid tissue in the cecal tonsils, increasing the number of FDC (del Cacho et al., 1993a), we isolated FDCs from infected chickens. Further studies are necessary to obtain a higher number of cells from normal chickens.

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